THE USE OF METABOLICALLY BLOCKED ORGANISMS FOR THE ANALYSIS OF BIOSYNTHETIC PATHWAYS[1]

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CONTENTS

In the field of cellular metabolism problems dealing with mechanisms of biosynthesis have been receiving an increasing share of attention during recent years. A major factor has been the growing understanding of the nature of energy relationships between catabolic and anabolic processes; this topic has been reviewed recently by Spiegelman and Sussman (45). Another broad aspect, and the one with which we are here concerned, is the question of biosynthetic pathways; *i.e.*, the chemical nature of the intermediates which are formed in the stepwise synthesis of complex cell components from simpler building block molecules.

I. EXPERIMENTAL APPROACHES TO THE PROBLEM

Three general approaches have been employed to obtain evidence concerning biosynthetic intermediates: the use of isotopic tracers; the use *in vitro* of enzyme systems; and the use *in vivo* of organisms with blocked biosynthetic pathways. In most metabolic reaction sequences the intermediates have a transitory existence and are present at any given moment in extremely small amounts. In general, the formation *in vivo* of a suspected intermediate in an uninterrupted sequence can only be verified by the technique of isotopic dilution. In order to use this technique, however, one must predict the structure of the intermediate and provide a pool of it, in the presence of which the organism is allowed to act on a primary labeled substrate; (or conversely the pool substance can be labeled and the primary substrate unlabeled). Following this action, the pool substance must be reisolated in pure form and analyzed for the presence of the other isotope. Another, less conclusive, tracer technique involves demonstrating that a suspected intermediate, when provided in labeled form, can be converted by the organism to the predicted labeled end product.

The second method, the use *in vitro* of enzyme preparations, is essential for confirmation of postulated biosynthetic reactions. However, like the tracer

[1] Unpublished experiments of the author referred to in this article were supported by a contract between the Regents of the University of California and the Office of Naval Research.

technique it can be used rarely as an exploratory tool since already inferred intermediates must be provided as substrates. Depending on the extent to which the enzyme system has been fractionated and on the experimental conditions, the *in vitro* preparation may serve either to demonstrate conversion of the substrate to the postulated end product or to convert it to a later intermediate whose structure can then be determined.

So far, the most informative technique has been to use *in vivo* blocks in biosynthetic pathways. As in the other two general methods, suspected intermediates can be tested; in this case the test consists of determining whether a given compound can reverse the block; ("reverse" in this discussion is used in the sense of evading or nullifying the block—not removing it). More important, however, is the fact that the experimentally induced deficiency often causes the accumulation of an intermediate, whose structure can then be ascertained for the first time.

Since the study of biosynthetic pathways is still largely in the exploratory stage, most of our knowledge in this field comes from experiments using the last of the three mentioned approaches. Experiments of this nature, dealing as they do with living organisms, pose many problems in interpretation, some of which will be discussed in the following paragraphs.

II. IN VIVO BLOCKS: TYPES OF EXPERIMENTAL EVIDENCE FOR PRECURSOR RELATIONSHIPS

The arguments to be presented apply to any organism in which the synthesis of a normal end product has been prevented, whether by mutation ("endogenous block"), addition of a chemical inhibitor ("exogenous block"), or by other means. The "normal end product" whose synthesis has been blocked can be a substance necessary for growth, such as an amino acid or a vitamin, or a substance with a dispensable or unknown function, such as a pigment or an antibiotic.[2]

Two general types of evidence can be obtained from studies of this nature. First, the absence of the normal end product may be associated with accumulation of a metabolite which in the unblocked organism is present in smaller (usually undetectable) amounts; it may be possible to obtain further information by experiments in which such an accumulation is stimulated by feeding the organism other compounds. Secondly, two or more compounds may be observed to reverse the block; *i.e.*, either permit growth or restore synthesis of an end product.

Each of these observations has been used at one time or another as evidence for a biosynthetic relationship; *i.e.*, as evidence that one compound is the precursor[3] of another. Thus, compound "A" has been deduced to be a precursor of compound "B" when, in a blocked organism:

(1) "B" restores growth, and "A" accumulates (Example 1A);

[2] In the following paragraphs, the term "dispensable end product" will be used to designate cellular products whose synthesis can be blocked without impairing the organism's ability to grow under the experimental conditions.

[3] By strictest definition, a "precursor" is a compound normally formed as an intermediate directly on the primary pathway of biosynthesis of the end product in question.

(2) "B", a dispensable end product, is no longer produced; "A" accumulates (Example 1B);

(3) "B" accumulates; this accumulation is significantly increased when "A" is added to the medium (Example 1C);

(4) Either compound "A" or compound "B" is capable of restoring growth (Example 1D);

(5) "B", a dispensable end product, is no longer synthesized; feeding "A" restores the ability to synthesize "B" (Example 1E).

Example 1

(A):

(1) *Endogenous block:* A *Neurospora* mutant using homocysteine for growth accumulates cystathionine (31). Conclusion:

→ cystathionine → homocysteine →

(2) *Exogenous block:* Purines reverse the block in sulfanilamide inhibited *Escherichia coli* (43). In the presence of sulfanilamide, aminoimidazolecarboxamide accumulates. Conclusion:

→ aminoimidazolecarboxamide → purine

(B) Chlorophyll is a "dispensable end product" for the alga *Chlorella*, growing in the dark in an organic medium. A mutation blocking chlorophyll production is associated with accumulation of protoporphyrin (22, 23). Conclusion:

→ protoporphyrin → chlorophyll

(C) A *Penicillium* mutant requiring biotin accumulates desthiobiotin. Adding pimelic acid to the medium increases thirtyfold the excretion of desthiobiotin (51). Conclusion:

→ pimelic acid → desthiobiotin →

(D):

(1) *Endogenous block:* A *Neurospora* mutant can use either α-aminoadipic acid or lysine for growth (36). Conclusion:

→ α-aminoadipic acid → lysine

(2) *Exogenous block:* Salicylate inhibition of *Escherichia coli* can be reversed by either pantoic acid or pantothenic acid (34). Conclusion:

→ pantoic acid → pantothenic acid

This excludes compounds which do not arise as intermediates endogenously but are convertible to a normal end product when supplied exogenously. For example, various analogues of thiazole were shown to be converted to the thiazole moiety of thiamin in the pea root (12) and, in a sense, behaved as precursors under the experimental conditions. However, they are not considered true biosynthetic intermediates and are excluded as true precursors by the above definition. Also excluded are compounds which arise by breakdown of the end product in question and are then able to be transformed back into that end product. For example, the keto acid corresponding to methionine arises endogenously in *Neurospora* by the action of L-amino acid oxidase on methionine (55). "Ketomethionine" can be enzymatically aminated back to methionine and hence can replace this amino acid for methionine deficient mutants (52); although it thus behaves like a precursor, it is not a normal intermediate in the primary biosynthesis of methionine in *Neurospora* (31) and hence is excluded by our definition.

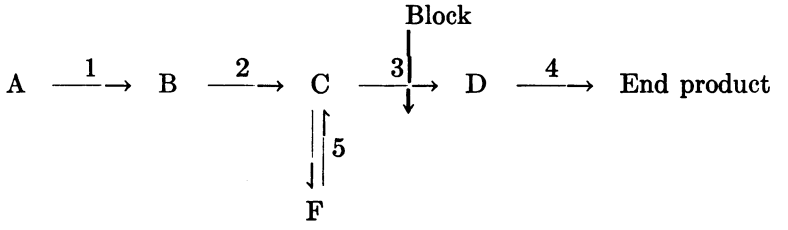
(E) Transplantation of imaginal discs from one eye-pigment mutant of *Drosophila* to another showed that a diffusible substance from the host restored synthesis of the normal red eye-pigment (7). The diffusible substance was later characterized as kynurenine (15). Conclusion:

→ kynurenine → red pigment

III. ALTERNATIVE INTERPRETATIONS OF THE EVIDENCE

For each of the five basic types of experimental evidence described in section II, only one interpretation has been mentioned thus far: namely, that a precursor relationship, as already defined, exists between the compounds involved. However, each type of experimental evidence *by itself* can support other equally tenable interpretations; these are presented in some detail below. For the sake of simplicity, the experimental types are grouped into three categories: absence of a normal end product correlated with the accumulation of a new compound; increased accumulation in response to the feeding of a different compound; and ability of two or more compounds to reverse the block.

A. *Accumulation correlated with a blocked synthesis:* Let us consider the case of a blocked biosynthesis of an essential end product; *i.e.*, a growth-factor deficiency resulting from mutation or exogenous inhibition. A compound found to accumulate under these conditions could occupy position "C" or "F" in the following diagram:



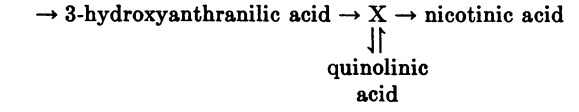
There is really no way to choose between these two possible interpretations. Generally, however, the investigator attempts an evaluation based on other evidence, such as might be obtained by growth studies, tracer experiments, the use of enzyme preparations, or considerations of chemical structures (Examples 2A and 2B). It is pertinent that, although the cases cited in Example 2 are com-

Example 2

(A) In certain mutant strains of *Neurospora* (3) and *E. coli* (2), a valine requirement is associated with the accumulation of α,β-dihydroxy-β-methylbutyric acid. Further evidence for assigning the latter a precursor role can be summarized as follows: the dihydroxy acid supports growth, at relatively high concentrations, of an earlier blocked *E. coli* mutant (3); it can be converted to the next valine intermediate, α-keto-β-methylbutyric acid, by cell-free extracts from wild type *E. coli* and *Neurospora* (2); its production by resting mycelia of appropriate double mutants of *Neurospora* is stimulated by the feeding of earlier precursors (2); and its structure is consistent with the mechanism postulated for its formation: methyl activated acetylation of α-hydroxy-β-ketobutyric acid followed by decarboxylation (53, 57). Conclusion:

→ α,β-dihydroxy-β-methylbutyric acid → α-keto-β-methylbutyric acid
↓
valine

(B) A nicotinic acid deficient mutant of *Neurospora* accumulates quinolinic acid (11). Quinolinic acid supports growth, at relatively high concentrations, of earlier blocked *Neurospora* mutants and of rats (27); it is formed enzymatically from 3-hydroxyanthranilic acid *in vitro* (9); it can be converted *in vivo* to nicotinic acid by *Neurospora* mycelia (62); and it has a chemically logical structure for an intermediate between 3-hydroxyanthranilic acid and nicotinic acid. Yanofsky and Bonner's conclusion, based on the high concentrations required for growth (11):



pletely parallel with respect to supporting evidence, one of the compounds in question (2A) was deduced to be a precursor, while the other (2B) was deemed a side product. These differences in interpretation stem entirely from differences of opinion regarding the significance of weak growth promoting ability. Actually, as illustrated in a later section, there are many situations in which a compound can be a true precursor and still exhibit weak or negative growth-stimulation.

In fact, negative data of any kind are particularly likely to be inconclusive when one is attempting to establish the role of an accumulated metabolite. A precursor may not, for example, be converted to a later intermediate or to the end product by unblocked strains because of impermeability in experiments *in vivo*, or because of enzyme inactivation or uncoupling in experiments *in vitro*. It may fail to show isotopic dilution in tracer studies because of nonmixing with the endogenously produced metabolite; it may fail to have a "logical" structure because of faulty logic on the part of the investigator.

What about the use of positive results as criteria? Referring again to the diagram, either "C" or "F" might support growth of organisms blocked at step 2; either "C" or "F" would be enzymatically convertible to "D" or to the end product (and as a corollary, an endogenously blocked mutant might be found to lack the enzyme system for converting either "C" or "F" to "D" or to the end product); either "C" or "F" might give positive results in any sort of tracer experiments; and either "C" or "F" might have structures appealing to the logic of the precursor hunter. Thus, the criteria that are generally offered in support of the view that an accumulated compound is indeed a true precursor are also equivocal.

The arguments presented for the case of accumulation associated with a growth-factor requirement apply equally to accumulations associated with blocked syntheses of dispensable end products. Example 3 illustrates a situation in which various accumulated carotenoids have been taken as carotene precursors by some investigators and as side products by others.

Example 3

In certain fungi (13, 20) and higher plants (42), when endogenous or exogenous blocks cause a decrease in the predominant carotene pigment, frequently there is observed a corresponding increase in ("accumulation of") more saturated carotenoids (*e.g.*, phytoene, phytofluene, *zeta*-carotene). On the basis of such observations Porter and Lincoln (42) have proposed a biosynthetic scheme for carotenoids involving stepwise dehydrogenation. Mackinney (35), however, points out that consideration of the *cis-trans* isomerism patterns makes such an assumption difficult, while Goodwin and Lijinsky (21) have suggested that

the various degrees of unsaturation may occur before, rather than after, polymerization of the isoprene units.

Thus, until further evidence is obtained, it cannot be decided whether the accumulated compounds represent intermediates in a series of dehydrogenation reactions or side products resulting from polymerization of variously saturated sub-units.

B. *Stimulation of accumulation by the feeding of other compounds:* In the diagram, let us assume a block at step 4 instead of at step 3, with accumulation of compound "D". Obviously, the addition of either compound "C" or "F" would increase the accumulation, so that again it is impossible to decide whether the active compound is a true precursor or a substance capable of conversion to a true precursor (see Example 4).

Example 4

The rat excretes N-methylnicotinamide in its urine.[4] This excretion is greatly stimulated by feeding the animal quinolinic acid (26). Since the latter is weak in growth promoting activity for nicotinic acid requiring strains of *Neurospora*, Yanofsky and Bonner assign it the role of a side product convertible to the true intermediate (see example 2B). Henderson and his co-workers (26), however, consider it a true intermediate in nicotinic acid biosynthesis:

→ 3-hydroxyanthranilic acid → quinolinic acid → nicotinic acid → nicotinamide
↓
N-methylnicotinamide

Still another interpretation may be considered in cases of this general type: namely, that the presence of high exogenous concentrations of the fed compound induces formation of enzymes which are normally absent or present in insignificant amounts [for a full discussion of this concept, see Monod and Cohn (38) and Foster (19)]. Ample precedent is found in experiments on catabolic reactions (Example 5), and this interpretation appears applicable in at least one biosynthesis investigation (Example 6).

Example 5

In cells of *Pseudomonas* sp. containing only normal amounts of endogenously synthesized tryptophan, this amino acid is partly converted to nicotinic acid and partly incorporated into protein. No oxidation of tryptophan to kynurenic acid or anthranilic acid can be detected. However, when such cells are placed in the presence of high exogenous concentrations of tryptophan, there is a tremendous stimulation of production of enzymes catalyzing the oxidation of tryptophan *via* kynurenine to kynurenic and/or anthranilic acids (48).

Example 6

Various strains of *Neurospora* accumulate anthranilic acid when suspended in a medium[5] containing high concentrations of tryptophan (24). The observation of this phenomenon

[4] Excretion (by a "normal" organism) of a cell constituent is a special case of accumulation behind a block. The "block" here consists of an inherent inability to use or break down the end product as rapidly as it is synthesized. This inability reflects a rate limiting reaction; *e.g.*, in the case in question the incorporation of nicotinamide into coenzyme I or II.

[5] A resting cell suspension or mycelium is again a "blocked" organism in that many of the syntheses involved in growth are prevented by the absence of some essential metabolite; various accumulations are now possible if "precursors" are fed.

in a mutant blocked at the conversion of anthranilic acid to tryptophan led Haskins and Mitchell to the conclusion that *Neurospora* employs a "tryptophan cycle". Bonner's group, however, showed by tracer experiments that the Haskins-Mitchell reaction does not operate in *Neurospora* when tryptophan is being synthesized endogenously in normal low amounts, and that the "cycle" is an artifact (40). The situation appears to the reviewer to be completely analogous to that described in Example 5, in that the "cycle" functions only when the medium contains concentrations of tryptophan sufficiently high to induce formation of kynureninase.

It is conceivable, also, that compound A might stimulate accumulation of compound B by virtue of being a coenzyme required for the synthesis of B, and originally present in rate limiting concentration (Example 7).

Example 7

Rats which are fed kynurenine have been shown to excrete much more N-methylnicotinamide if they have a normal intake of vitamin B_2 than if they are deficient for this vitamin (28). The interpretation is not that vitamin B_2 is a precursor of nicotinic acid, however, but rather that it is the coenzyme for a step in the synthesis of the latter compound:

$$\rightarrow \text{kynurenine} \xrightarrow{B_2} \text{3-hydroxykynurenine} \rightarrow \text{3-hydroxyanthranilic acid} \rightarrow \text{nicotinic acid} \rightarrow$$

Negative results are equally ambiguous: the failure of compound "A" to stimulate accumulation of "B" must be regarded with suspicion in all cases for the reasons discussed below concerning negative results in nutritional studies.

C. *Ability of compounds to permit growth or restore synthesis:* When a growth-factor requirement is induced, endogenously or exogenously, and two different compounds are each capable of restoring growth, a sequential relationship between them may indeed be involved. If both compounds have structures known to occur in protoplasm, however, it is no simple matter to decide from this evidence which of the two is formed earlier in the sequence (Example 8). In most cases, however, only one of the compounds in question is a normal cell constituent and can be assigned the role of end product with relative safety. Whether other compounds permitting growth are true precursors or not is quite another matter. There is the question of positions "C" and "F" in the above diagram, for example. If the organism is blocked at step 2 instead of at step 3, it can readily be seen that either "C" or "F" will serve in place of the end product to restore growth (Example 9).

Example 8

A lactic acid bacterium can use either histidine or purines for growth. Experiments performed with this organism led Broquist and Snell (14) to the conclusion that the purines were acting as precursors to histidine although it had previously been believed that histidine was a precursor of purines. Tracer experiments with yeast (33) and the rat (54) have failed to confirm either relationship, however, so that the significance of the *Lactobacillus* experiments is doubtful. Recent experiments with *Neurospora* indicate that the carbon chain of histidine may be derived from ribose (5).

Example 9

α-Hydroxy-β-ethylbutyric acid can replace isoleucine for the growth of certain bacterial mutants (1); this is undoubtedly accomplished through oxidation to the α-keto

compound since Snell's group (30) has shown that in other bacteria the ability to use an α-hydroxy compound in place of the corresponding amino acid for growth is correlated with the presence in the cells of a suitable dehydrogenase. In the case of isoleucine biosynthesis, however, the sequence shown below is well enough established to rule out the monohydroxy acid as a true biosynthetic intermediate (56). Conclusion:

$$\rightarrow \alpha,\beta\text{-dihydroxy-}\beta\text{-ethylbutyric acid} \rightarrow \alpha\text{-keto-}\beta\text{-ethylbutyric acid} \rightarrow \text{isoleucine}$$
$$\uparrow$$
$$\alpha\text{-hydroxy-}\beta\text{-ethylbutyric acid}$$

Another interpretation occasionally arising from experiments of this general type is that one of the compounds restoring growth is a coenzyme in the production of the other. (The missing end product in this case is the coenzyme; it can be dispensed with if all the end products of its action are supplied.) Sometimes a relationship of this type is clearly indicated on the basis of relative concentrations required for half-maximal growth (Example 10); however, even such quantitative considerations can be misleading (Example 11), and in some cases they cannot be applied (Example 12). The case cited in Example 12 is particularly interesting because it introduces the concept that two compounds might reverse a block, not because one is convertible to the other, but because either can perform the needed function.

Example 10

Certain bacterial strains are known to require either catalytic amounts of vitamin B_{12} or much larger amounts of desoxyribonucleotides for growth (61); B_{12} is here interpreted as a coenzyme involved in nucleotide synthesis.

Example 11

A *Neurospora* mutant requires either catalytic amounts of nicotinic acid or much larger amounts of tryptophan for growth (8); according to the line of reasoning used in Example 10, nicotinic acid would appear to be a catalyst for tryptophan formation. Indeed, this interpretation was suggested by Beadle, Mitchell and Nyc (8). However, the fact that tryptophan is a precursor of nicotinic acid (25) has suggested other interpretations of this mutant's requirements:

(a) Davis (16) proposed that this mutant may have a partial block prior to tryptophan, limiting the amount of tryptophan synthesized. Under such conditions the tryptophan might preferentially be utilized for protein synthesis, reducing to negligible the amount of tryptophan entering the nicotinic acid pathway. In such a situation, the balance would be restored by the addition of more tryptophan; the mutant would also grow if nicotinic acid itself were added.

(b) Newmeyer and Tatum (39) have obtained isolates of this strain from back-crosses to wild type that grow very poorly on tryptophan and suggest that the strain is actually blocked between tryptophan and nicotinic acid. The addition of small amounts of nicotinic acid alone would support growth since, according to this hypothesis, the synthesis of tryptophan is unimpaired; a large amount of tryptophan would restore nicotinic acid synthesis by somehow overcoming the block.

The mechanism of tryptophan's reversing action would depend on the nature of the block, regarding which no evidence is presented. It is conceivable, however, that large amounts of an intermediate could overcome a block later in the pathway if the block were due to production of a competitive inhibitor or to establishment of a reaction removing the intermediate by another pathway.

Example 12

Certain bacteria have been found to show an alternative requirement for biotin or oleic acid (60). Two interpretations of this evidence were:

(a) Biotin is a coenzyme in oleic acid biosynthesis (oleic acid was the only end product of biotin action omitted from the growth medium) (60).

(b) The organism requires a surface active agent; either biotin or oleic acid can fulfill this requirement (59).

In other cases of alternative requirements, much more indirect relationships are indicated (Examples 13 and 14). These examples concern growth promoting compounds (bromouracil and sulfanilamide) whose chemical structures immediately suggest abnormal functions. The existence of such examples, however, suggests that similar phenomena may underlie *any* case of alternative growth requirements, even when the compounds concerned have plausible structures.

Example 13

Lactobacillus casei, when in a folic acid medium, can be inhibited by the addition of 5-nitrouracil ("exogenous block"). Growth is restored by addition of either thymine or 5-bromouracil (29). The investigators do not, however, infer that 5-bromouracil is a precursor of thymine. In fact, they note that this situation serves as a warning that "inferences and deductions drawn from the action of secondary reversing agents should be regarded as suggestions only and not as definitive conclusions."

Example 14

Two *Neurospora* mutants are known which require sulfanilamide or threonine (63) and sulfanilamide or methionine (17), respectively. That sulfanilamide plays a normal role in metabolism is unlikely, however. Zalokar (63) has presented convincing evidence that the former mutant uses normal levels of *p*-aminobenzoic acid to catalyze a deleterious reaction not characteristic of wild type *Neurospora*; the end product of this reaction is thought to be a competitive inhibitor of threonine utilization. Thus, threonine supports growth since it is the competitive normal metabolite, but the growth stimulation by sulfanilamide is attributed to its interference with the PAB-catalyzed synthesis of the inhibitor. The sulfanilamide–methionine mutant is believed to differ only in the relative sensitivities of methionine and threonine metabolism.

It is particularly difficult to come to a positive conclusion if the nature of the block itself is not known with certainty. As in the sulfa requiring mutants (Example 14) mutational blocks may be caused by the production of inhibitors rather than by deletion of enzymes, and at least one example has been described in which the growth-factor requirement of a mutant probably resulted from increased activity of an enzyme catalyzing destruction of the essential product (32). Without positive evidence, then, the block itself may be due to any of several different phenomena, each subject to its own peculiar reversal mechanisms.

As mentioned earlier, negative results are particularly likely to be inconclusive; a compound may be a true precursor, yet fail to support growth in place of the end product for a variety of reasons. These include: chemical instability (Example 15), toxicity (Examples 16 and 17), and inability to penetrate the cell (Example 18). In studies of catabolism there are enough well substantiated examples of permeability difficulties preventing demonstration *in vivo* of enzymes

known to function *in vitro* [see, for example, Seaman and Houlihan (44) and Stanier (47)] to necessitate giving this phenomenon serious consideration whenever one is dealing with failures to reverse *in vivo* blocks.

Example 15

Bonner was unable to satisfy the requirement of the rat for nicotinic acid by feeding 3-hydroxyanthranilic acid (10). This was later shown to be caused by the instability of the latter compound; much larger doses were found completely effective (37).

Example 16

Tubercle bacilli require certain long-chain fatty acids for maximal growth. However, these must be either esterified or mixed with serum albumin, otherwise they strongly inhibit the organism that requires them (18).

Example 17

Balis *et al.* (6) found that labeled 2,6-diaminopurine is extensively converted to polynucleotide adenine and guanine by *Lactobacillus casei*, even under conditions such that the diamino compound is acting as a growth inhibitor.

Example 18

α,β-Dihydroxy-β-methylbutyric acid is a relatively strong acid. It can be demonstrated to have valine replacing activity for *E. coli* mutants, but only below pH 6.0 (2); presumably this is due to impermeability of the cell toward the ionized acid. At such low pH values, however, growth is suboptimal and quantitative comparisons are not valid.

Finally, there is the obvious consideration that a compound may fail to reverse the block because the block is acting at a later step; *i.e.*, "D" but not "C" will reverse the block in the diagram above.

Although in the foregoing discussion all examples have had to do with growth-factor requirements, it goes without saying that the same arguments apply to situations involving blocked syntheses of dispensable end products. In other words, a compound may act to restore such a synthesis by a catalytic effect, by indirect action, or through conversion to a normal intermediate. Negative results may be due to the same phenomena discussed for growth-factor experiments.

IV. CONCLUSIONS

While each type of evidence, by itself, is subject to diverse interpretations, the number of these can be reduced to a minimum by combining as many different approaches as possible. For example, interconversions can be confirmed by appropriate tracer studies, and the bearing of permeability on the results can be ascertained by enzyme experiments *in vitro*. Actually, there exist today a great many instances in which a proposed precursor has satisfied all the criteria of accumulation, growth stimulation, enzymatic conversion *in vitro*, tracer conversion *in vivo*–in fact, all the types of experimental evidence discussed in this paper. Nevertheless, in no case have the results completely ruled out the possibility that the compound in question is a side product in enzymatic equilibrium with the true precursor.

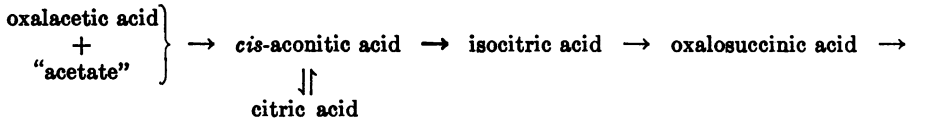
In theory, however, a criterion exists which would be truly conclusive: if a single enzyme could be shown to catalyze the conversion of "B" to "X" and another single enzyme could be shown to convert "X" to "D", compound "X" would be established with finality as the true intermediate between "B" and "D" (occupying position "C" and not "F" in the diagram, since "B" to "F" and "F" to "D" each require a mixture of two enzymes to proceed).

In this case, the strength of the proof is limited solely by the reliability with which it is known that one is dealing with a "single enzyme". In modern enzymology, enzyme purity is judged on the basis of crystallinity and homogeneity in the ultracentrifuge and electrophoresis cell; however, these criteria cannot determine absolute purity since, as Pirie points out (41), two species of protein molecules will not be separable if they differ in only a few chemical groups. Furthermore, even if absolute purity of enzymes were attainable, the reconstructed sequence of reactions which such enzymes catalyzed would be established only as an *in vitro* phenomenon; there would be no assurance that the same sequence took place *in vivo*.

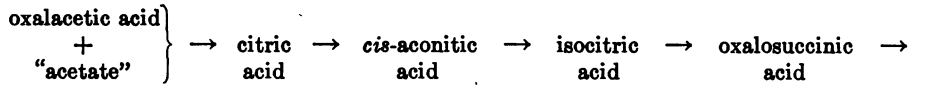
The "truly conclusive" criterion for establishment of a biosynthetic[6] intermediate thus exists in theory only; we are left with the conclusion that present-day techniques are unable to establish metabolic intermediates with finality. In practice, however, we do rely on the "single enzyme" concept as the ultimate in positive evidence (Example 19), with crystallinity as the test of enzyme purity. Unfortunately, biosynthetic investigations have not even reached the point of dealing commonly with crystalline enzymes so that the existing body of schemes for biosynthesis is based on less satisfactory types of evidence.

Example 19

In the tricarboxylic acid cycle, the condensation of oxalacetic acid and "active acetate" was believed, on the basis of indirect evidence, to result in the formation of *cis*-aconitic acid; citric acid was judged to be a side product in enzymatic equilibrium:



Recently, the isolation in crystalline form of the enzyme condensing oxalacetate with coenzyme A linked acetate (50) established the product of the reaction to be citric acid itself. Since the above scheme demands the function of two enzymes for citric acid formation from these substrates, it has been corrected as follows (35a):



[6] It should be noted that the exact set of arguments apply whether the series "A → → → end product" is a biosynthetic or a catabolic sequence. Even the technique of "simultaneous adaptation" (46) [used generally in catabolism studies, though applicable also to biosynthetic problems (58)] cannot distinguish between the two possibilities since two compounds in enzymatic equilibrium will both give positive results by this method (4, 49).

The foregoing remarks are not meant to imply, however, that until we can work with enzymes of crystalline purity it would be better to abandon our investigations on biosynthesis. On the contrary, it is useful, satisfying and important to know that "A" accumulates when "B" 's synthesis is blocked, that labeled "A" can be traced to labeled "B", or that "A" can replace "B" in growth-factor experiments. The scheme arrived at for the biosynthesis of nicotinic acid, for example, is a brilliant contribution to our knowledge of intermediary metabolism, and it is not the purpose of this article to detract from such achievements in any way. It is, however, hoped that this discussion may help those working in other fields to evaluate better the evidence offered in support of biosynthetic schemes and to form from them their own objective interpretations.

V. SUMMARY

A large share of our knowledge of biosynthetic mechanisms comes from experiments with organisms either chemically or genetically blocked *in vivo*. The evidence obtained usually takes the form of observations regarding accumulated products or the ability of added compounds to reverse the block. Positive results are frequently presented as evidence of true precursor relationships; it has been the purpose of this paper to document cases which clearly illustrate that for each type of experiment there are other, equally tenable interpretations. Most of these alternative interpretations can be eliminated by the proper combination of techniques. However, with present day techniques it is not possible to establish unequivocally that a given compound is a true intermediate and not an enzymatically interconvertible side product.

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